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Ethyl pyruvate modulates inflammatory gene expression in mice subjected to hemorrhagic shock

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Yang, Runkuan, David J. Gallo, Jeffrey J. Baust, Takashi Uchiyama, Simon K. Watkins, Russell L. Delude, and Mitchell P. Fink. Ethyl pyruvate modulates inflammatory gene expression in mice subjected to hemorrhagic shock. *Am J Physiol Gastrointest Liver Physiol* 283: G212–G221, 2002. First published February 27, 2002; 10.1152/ajpgi.00022.2002.—Administration of pyruvate, an effective scavenger of reactive oxygen species, has been shown to be salutary in numerous models of redox-mediated tissue or organ injury. Pyruvate, however, is unstable in solution and, hence, is not attractive for development as a therapeutic agent. Herein, ethyl pyruvate, which is thought to be more stable than the parent compound, was formulated in a calcium-containing balanced salt solution [Ringer ethyl pyruvate solution (REPS)] and evaluated in a murine model of hemorrhagic shock and resuscitation (HS/R). Resuscitation with REPS instead of Ringer lactate solution (RLS) significantly improved survival at 24 h and abrogated bacterial translocation to mesenteric lymph nodes and the development of increased ileal mucosal permeability to FITC-labeled dextran (4,000 Da) at 4 h. Mice treated with REPS instead of RLS also had lower circulating levels of alanine aminotransferase at 4 h. Treatment with REPS instead of RLS decreased activation of nuclear factor-κB in liver and colonic mucosa after HS/R and also decreased the expression of inducible nitric oxide synthase, tumor necrosis factor, cyclooxygenase-2, and interleukin-6 mRNA in liver, ileal mucosa, and/or colonic mucosa. These data support the view that resuscitation with REPS modulates the inflammatory response and decreases hepatocellular and gut mucosal injury in mice subjected to HS/R.

translocation; bacterial; permeability; mucosal; tumor necrosis factor; cyclooxygenase-2; inducible nitric oxide synthase

REACTIVE SPECIES OF OXYGEN (ROS) have been implicated as being important mediators in a variety of pathological conditions, including burns (20), various forms of ischemia-reperfusion (I/R) injury (8, 15, 16, 26), and hemorrhagic shock (9, 10, 17, 32). Examples of ROS of biological importance include superoxide radical anion ($O_2^-\cdot$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH\cdot$), and peroxynitrite ($ONOO^-$). Although $O_2^-\cdot$ is only moderately reactive, it can be converted by the enzyme superoxide dismutase to the more reactive species H_2O_2. Both $O_2^-\cdot$ (37) and H_2O_2 (24) are capable of reacting with nitric oxide to form the highly reactive moiety $ONOO^-$. In addition, in a series of reactions catalyzed by ionized iron, $O_2^-\cdot$ and H_2O_2 can interact to form another extremely reactive species, $OH\cdot$ (12).

Pyruvate, a key intermediate in cellular metabolism, is an effective scavenger of ROS. In a reaction characteristic of α-keto carboxylic acids in general, pyruvic acid (the simplest member of this class of compounds) rapidly undergoes nonenzymatic decarboxylation in the presence of H_2O_2 to form acetate, carbon dioxide, and water (5, 33). Recently, pyruvate also has been shown to be capable of scavenging $OH\cdot$ (11). Administration of exogenous pyruvate has been shown to be salutary in numerous models of redox-mediated tissue or organ injury (6–8, 39).

Despite these promising findings, the usefulness of pyruvate as a therapeutic agent is limited by its poor stability in solution (54). When dissolved in an aqueous solvent, pyruvate spontaneously undergoes condensation and cyclization reactions to form a variety of products, some of which may be toxic (31). To circumvent this issue, Sims et al. (48) formulated a derivative of pyruvic acid, namely ethyl pyruvate, in a calcium- and potassium-containing balanced salt solution. These investigators showed that treatment with this fluid, called Ringer ethyl pyruvate solution (REPS), ameliorates structural and functional damage to the intestinal mucosa caused by mesenteric I/R in rats. Subsequently, Tawadrous et al. (51) showed that resuscitation with REPS instead of Ringer lactate solution (RLS) prolongs survival and decreases intestinal mucosal injury in rats subjected to hemorrhagic shock. In this study, Tawadrous et al. (51) also obtained biochemical evidence that resuscitation with REPS instead of RLS ameliorates hepatic and intestinal mucosal lipid peroxidation. These findings support the view that ethyl pyruvate is an effective ROS scavenger.

ROS have been implicated in the activation or modulation of a number of important intracellular signal transduction pathways, including signaling mediated

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by the transcription factor nuclear factor (NF)-κB (52). Activation of the NF-κB pathway is important in regulating the expression of a number of genes involved in the inflammatory response, including inducible nitric oxide synthase (iNOS) (36), cyclooxygenase (COX)-2 (19), tumor necrosis factor (TNF) (27), and interleukin (IL)-6 (1, 23, 40, 45). Recent work by several groups has shown that many of these genes are activated in mice subjected to hemorrhagic shock and resuscitation (HS/R) (13, 46, 47, 50).

In view of the foregoing, the goal of the present investigation was to gain additional information about the effects of infusing REPS instead of RLS to resuscitate experimental animals from hemorrhagic shock. Specifically, we sought to test the hypothesis that treatment with REPS would blunt activation of NF-κB signaling and the activation of several inflammatory genes in three organs: the liver, the ileum, and the colon. In these studies, we show that treatment with REPS ameliorated HS/R-induced hepatocellular injury and gut barrier dysfunction and also downregulated the inflammatory response associated with resuscitation from hemorrhagic shock.

MATERIALS AND METHODS

This research protocol complied with the regulations regarding animal care as published by the National Institutes of Health and was approved by the Institutional Animal Use and Care Committee of the University of Pittsburgh Medical School. Male C57BL/6 mice weighing 20–25 g (Jackson Laboratories, Bar Harbor, ME) were used in this study. The animals were maintained at the University of Pittsburgh Animal Research Center with a 12:12-h light-dark cycle and free access to standard laboratory feed and water. Animals were not fasted before the experiments. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Experimental designs for animal experiments. The shock model employed has been described previously (17). Briefly, mice were anesthetized by intramuscular pentobarbital sodium (90 mg/kg). Both femoral arteries were surgically prepared and cannulated. The left artery was used for continuous blood pressure monitoring. The right artery was used for blood withdrawal and blood and fluid administration. The mice were subjected to hemorrhagic shock by withdrawal of blood (2.25 ml/100 g body wt) over 10 min to achieve a mean arterial pressure (MAP) of 30 mmHg. MAP was maintained at 30 mmHg for 2.0 h with continuous monitoring of blood pressure and withdrawal and return of blood as needed. Cannulas, syringes, and tubing were flushed with heparin sodium (1,000 U/ml) before all procedures. The animals were resuscitated to an initial MAP ≥ 80 mmHg by administration of all remaining shed blood plus intra-arterial injection of 2× shed blood volume of either RLS or REPS over 30 min. Sham controls were subjected to the same anesthetic and cannulation procedures but were not subjected to hemorrhagic shock. The composition of RLS was as follows (in mM): 109 NaCl, 4.0 KCl, 2.7 $CaCl_2$, and 28 sodium lactate. The composition of REPS was as follows (in mM): 130 NaCl, 4 KCl, 2.7 $CaCl_2$, and 28 ethyl pyruvate.

In *experiment A*, we studied three groups of mice: controls (Sham, $n = 7$), hemorrhagic shock plus resuscitation with RLS (RLS, $n = 15$), and hemorrhagic shock plus resuscitation with REPS (REPS, $n = 8$). Four hours after resuscitation (or sham shock), surviving mice were reanesthetized with intramuscular pentobarbital sodium (90 mg/kg), and the following procedures were performed: a segment of ileum was harvested for determination of mucosal permeability; the mesenteric lymph node (MLN) complex was harvested to measure bacterial translocation; blood was aspirated from the heart to measure the plasma concentration of alanine aminotransferase (ALT); and portions of the liver, colon, and ileum were harvested for determination of NF-κB activation using electrophoretic mobility shift assay (EMSA) and expression of proinflammatory genes using semiquantitative RT-PCR. In addition, portions of the liver were collected to evaluate iNOS protein expression by immunohistochemistry.

Experiment B was carried out to compare the effects of resuscitation with RLS vs. REPS on survival. Mice were subjected to HS/R (or the sham procedure) as described above, and survival was assessed at 24 h.

Intestinal mucosal permeability. Intestinal mucosal permeability to the fluorescent tracer fluorescein isothiocyanate-dextran with a molecular mass of 4,000 Da (FD-4) was determined using an everted gut sac method, as previously described by Wattanasirichaigoon et al. (55). Everted gut sacs were prepared in ice-cold modified Krebs-Henseleit bicarbonate buffer (KHBB, pH 7.4). One end of the gut segment was ligated with a 4-0 silk suture. The segment was then everted onto a thin plastic rod, and the resulting gut sac was secured with a 4-0 silk suture to the grooved tip of a 3-ml plastic syringe containing KHBB. The sac was gently distended by injecting 1.5 ml of KHBB. The sac was suspended in a 50-ml beaker containing 40 ml of KHBB plus FD-4 (40 mg/ml). The solution in the beaker was temperature jacketed at 37°C and was continuously bubbled with a gas mixture containing 95% O_2-5% CO_2. We took a 1.0-ml sample from the beaker before putting in the gut sac to determine the initial external (i.e., mucosal surface) FD-4 concentration. The sac was incubated for 30 min in the KHBB solution containing FD-4. The length of the gut sac was measured. Fluid from the inside of the sac was aspirated for the determination of FD-4 concentration. The serosal and mucosal samples were centrifuged for 10 min at 1,000 *g*. The supernatant (300 μl) was diluted with phosphate-buffered saline (PBS, 2.7 ml). Fluorescence was measured using a Perkin-Elmer LS-50 fluorescence spectrophotometer (Palo Alto, CA) at an excitation wavelength of 492 nm (slit width, 2.5 nm) and an emission wavelength of 515 nm (slit width, 10 nm). Permeability was expressed as the mucosal-to-serosal clearance of FD-4 as previously described (55).

Bacterial translocation. The skin was cleaned with 10% povidone iodine. Using sterile technique, we opened the abdominal cavity and exposed the viscera. The MLN complex was removed, weighed, and placed in a grinding tube containing 0.5 ml of ice-cold PBS. The MLN were homogenized with glass grinders, and a 250-μl aliquot of the homogenate was plated onto brain-heart infusion and MacConkey agar (Becton Dickinson, Franklin Lakes, NJ). The plates were examined 24 h later after being aerobically incubated at 37°C. The colonies were counted and results expressed as colony-forming units (CFU) per gram of tissue.

Serum ALT measurement. Blood (200 μl) was obtained by cardiac puncture and placed in a 0.5-ml centrifugation tube on ice. The samples were then centrifuged at 5,000 *g* for 3 min. The serum was collected and assayed for ALT using an automated assay system.

Immunohistochemistry for iNOS expression. Tissues were fixed in 2% paraformaldehyde for 1 h, then treated with 30% sucrose overnight. Fixed sections were washed three times in PBS containing 0.5% BSA and 0.15% glycine, pH 7.4 (*buffer*

A). The fixed and washed sections were incubated for 30 min with purified goat IgG (50 mg/ml) at 25°C and then washed three more times with *buffer A*. All the preceding steps were designed to ensure minimal nonspecific reaction to the antibodies used. Sections were then incubated for 60 min with a primary antibody to iNOS (1 μg/ml; Transduction Laboratories, Newcastle, UK). This step was followed by three washes in *buffer A* and a 60-min incubation with a fluorescently labeled second antibody (Alexa 488, 1–2 mg/ml; Molecular Probes, Eugene, OR) mixed with *buffer A*. The sections were then washed six times (5 min/wash) in *buffer A*, washed for 1 min in PBS containing 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), an ultraviolet-excited DNA stain to delineate nuclei, and then mounted in gelvatol and coverslipped for light microscopy. Observation was with an Olympus Provis microscope equipped with a cooled charge-coupled device camera.

Assessment of NF-κB activation. To prepare nuclear extracts, we homogenized murine tissue samples with T-PER (Pierce, Rockford, IL), using a 1:20 ratio of tissue-to-sample preparation reagent, as directed by the manufacturer's instructions. The samples were centrifuged at 10,000 *g* for 5 min to pellet tissue debris. The supernatant was collected and frozen at −80°C. Nuclear protein concentration was determined using a commercially available Bradford assay (Bio-Rad, Hercules, CA).

The EMSA assay for NF-κB nuclear binding was performed using a duplex oligonucleotide probe based on the NF-κB binding site upstream of the murine iNOS promoter. The sequence of the double-stranded NF-κB oligonucleotide was as follows: sense, 5′-AGT TGA GGG GAC TTT CCC AGG C-3′; antisense, 3′-TCA ACT CCC CTG AAA GGG TCC G-5′ (Promega, Madison, WI). The oligonucleotides were end labeled with adenosine [γ-^{32}P]triphosphate (New England Nuclear, Boston, MA) using T4 polynucleotide kinase (Promega). Nuclear protein (6 μg) was incubated at room temperature with γ-^{32}P-labeled NF-κB probe in 4 μl of 5× binding buffer (65 mM HEPES, 325 mM NaCl, 5 mM dithiothreitol, 0.7 mM EDTA, and 40% glycerol, pH = 8.0) in the presence of 2 μg of poly(DI-DC) for 20 min, the total volume of the binding reaction mixture being 20 μl. The binding reaction mixture was electrophoresed on 4% nondenaturing PAGE gels. After electrophoresis, the gels were dried and exposed to Kodak (Rochester, NY) X-Omat film at −80°C. The autoradiograms were quantified by scanning densitometry.

To determine the specificity of binding reactions, we carried out "cold competition" studies using a 100-fold molar excess of either unlabeled NF-κB duplex oligonucleotide (specific competition) or an irrelevant oligonucleotide probe. For the latter, we used a probe containing the hypoxia-inducible factor (HIF)-1 binding sequence from the human erythropoeitin 3′ enhancer. Supershift assays were performed by incubating nuclear extracts with 2 μl of anti-p65 and anti-p50 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h before the addition of radiolabeled probe. The binding reaction mixture was electrophoresed on 4% PAGE gels. After electrophoresis, the gels were dried and exposed to XAR-5 film (Kodak) at −80°C for overnight using an intensifying screen.

RT-PCR. Total RNA was extracted from harvested tissues with chloroform and TRI reagent (Molecular Research Center, Cincinnati, OH) exactly as directed by the manufacturer. We treated the total RNA with DNAFree (Ambion, Houston, TX) as instructed by the manufacturer, using 10 U DNase I/10 μg RNA. Two micrograms of total RNA were reverse transcribed in a 40-μl reaction volume containing 0.5 μg oligo(dT)$_{15}$ (Promega), 1 mM of each dNTP, 15 units avian myeloblastosis virus RT (Promega), and 1 U/μl recombinant RNasin ribonuclease inhibitor (Promega) in 5 mM $MgCl_2$, 10 mM Tris·HCl, 50 mM KCL, and 0.1% Triton X-100 (pH 8.0). The reaction mixtures were preincubated at 21°C for 10 min before DNA synthesis. The RT reactions were carried out for 50 min at 42°C and were heated to 95°C for 5 min to terminate the reaction. Reaction mixtures (50 μl) for PCR were assembled using 5 μl cDNA template, 10 units AdvanTaq Plus DNA polymerase (Clontech, Palo Alto, CA), 200 μM of each dNTP, 1.5 mM $MgCl_2$, and 1.0 μM of each primer in 1× AdvanTaq Plus PCR buffer. PCR reactions were performed using a model 480 thermocycler (Perkin-Elmer, Norwalk, CT). Amplication was initiated with 5 min of denaturation at 94°C. The PCR conditions for amplifying cDNA for TNF, IL-6, and COX-2 were as follows: denaturation at 94°C for 45 s, annealing at 61°C for 45 s, and polymerization at 72°C for 45 s. To ensure amplification was in the linear range, we empirically identified the optimal number of cycles as 33, 35, and 35 for TNF, IL-6, and COX-2, respectively. Amplification of cDNA for iNOS was carried out by denaturing at 94°C for 45 s, annealing at 58°C for 1 min, and polymerizing at 72°C for 45 s for 35 cycles. This number of PCR cycles was empirically determined to ensure that amplification was in the linear range. After the last cycle of amplification, the samples were incubated in 72°C for 10 min and then held at 4°C. The 5′ and 3′ primers for iNOS were CAC CAC AAG GCC ACA TCG GAT T and CCG ACC TGA TGT TGC CAT TGT T, respectively (Invitrogen, Carlsbad, CA); the expected product length was 426 bp. The 5′ and 3′ primers for TNF were GGC AGG TCT ACT TTG GAG TCA TTG C and ACA TTC GAG GCT CCA GTG AAT TCG G, respectively; the expected product length was 307 bp. The 5′ and 3′ primers for IL-6 were CTG GTG ACA ACC ACG GCC TCC CCT and ATG CTT AGG CAT AAC GCA CTA GGT, respectively; the expected product length was 600 bp. The 5′ and 3′ primers for COX-2 were GTC TGA TGA TGT ATG CCA CAA TCT G and GAT GCC AGT GAT AGA GGG TGT TGA A, respectively; the expected product length was 276 bp. 18S ribosomal RNA was amplified to verify equal loading. For this reaction, the 5′ and 3′ primers were CCC GGG GAG GTA GTG ACG AAA AAT and CGC CCG CTC CCA AGA TCC AAC TAC, respectively; the expected product length was 200 bp. Ten microliters of each PCR reaction were electrophoresed on a 2% agarose gel, scanned at a NucleoVision imaging workstation (NucleoTech, San Mateo, CA), and quantified using GelExpert release 3.5.

Statistical methods. Results are presented as means ± SE. Differences in CFU between groups were analyzed using the Wilcoxon *U*-test. Other continuous data were analyzed using Student's *t*-test or analysis of variance followed by Fisher's least significant differences test as appropriate. Survival data were analyzed using the χ^2 test. P values < 0.05 were considered significant. Summary statistics are presented for RT-PCR data, but these results were not subjected to statistical analyses, since the method employed was only semiquantitative and the samples sizes (n = 3–4) were small.

RESULTS

Survival. Experiment A was not designed as a survival study. The mice were intentionally killed 4 h after the end of resuscitation to assess intestinal barrier function and to obtain blood and tissue samples for determinations of plasma ALT concentration, NF-κB activation, and steady-state mRNA levels for several genes. Nevertheless, in *experiment A*, all of the mice in

Table 1. *Effect of resuscitation with REPS on survival in mice subjected to HS/R*

	Experiment					
	A			B		
	Survival at 4 h (%)	*P* Value vs. Sham	*P* Value vs. RLS	Survival at 24 h (%)	*P* Value vs. Sham	*P* Value vs. RLS
Sham	7/7(100)			10/10(100)		
RLS	12/15(80)	NS		5/10(50)	0.01	
REPS	8/8(100)	NS	NS	11/12(92)	NS	0.029

RLS, mice treated with hemorrhagic shock plus resuscitation (HS/R) with Ringer lactate solutions; REPS, mice treated with HS/R with Ringer ethyl pyruvate solution; Sham, control mice; NS, not significant.

the Sham and REPS groups survived until they were killed at 4 h after resuscitation (Table 1). In contrast, only 12 of 15 mice in the RLS group survived to the 4-h postresuscitation time point. *Experiment B* was performed as a survival study. In this experiment, all of the mice in the Sham group and 11 of 12 mice in the REPS group survived for at least 24 h, whereas 5 of 10 mice in the RLS group died before the 24-h postresuscitation time point. At 24 h, all of the surviving mice in the Sham and REPS groups were active and eating and drinking normally.

Intestinal barrier dysfunction. In the RLS group, ileal mucosal permeability to FD-4 was approximately twofold greater than in the Sham group (Fig. 1*A*). However, resuscitation with REPS instead of RLS significantly ameliorated the increase in mucosal permeability induced by the HS/R protocol. Bacterial translocation to MLN was minimal in sham-operated controls but was extensive in the RLS group (Fig. 1*B*). Resuscitation with REPS virtually abrogated bacterial translocation after HS/R.

Hepatocellular damage. The mean plasma ALT concentration was significantly greater in the RLS group than in the sham-operated control group (Fig. 2). However, the mean circulating level of this marker of hepatocellular injury was significantly lower in the REPS group than in the RLS group.

NF-κB activation. We used EMSA to detect the transcription factor NF-κB in nuclear extracts prepared from samples of liver and ileal and colonic mucosa obtained from mice 4 h after resuscitation from hemorrhagic shock with either RLS or REPS. We also assayed nuclear extracts prepared from samples obtained from control animals (Sham group) that were anesthetized and cannulated but not subjected to shock. There was evidence of basal DNA binding of NF-κB in all the tissues examined, but especially in samples of ileal mucosa (Fig. 3*A*). After resuscitation with RLS, NF-κB DNA binding increased in liver and colonic mucosa but was unchanged in ileal mucosa. To confirm the identity of the activated protein-DNA complex, we carried out binding assays with samples that were preincubated with specific antibodies directed against p50 and p65. Although we failed to observe a supershift with the anti-p50 antibody, we observed both a supershifted band and decreased intensity of the NF-κB band with the p65 antibody. Moreover, binding of the protein to labeled NF-κB binding element was completely inhibited by a 100-fold excess of unlabeled NF-κB duplex oligonucleotide but not by a similar molar excess of unlabeled HIF-1 duplex oligonucleotide.

NF-κB DNA binding was decreased in hepatic tissue and colonic mucosal samples from mice in the REPS group compared with hepatic and colonic mucosal samples from mice in the RLS group (Fig. 3*B*).

Expression of stress-related genes. Hepatic iNOS, TNF, and COX-2 mRNA expression clearly increased after HS/R in the RLS group (Fig. 4, *A–C*, respectively). Resuscitation with REPS instead of RLS decreased post-HS/R expression of all three genes.

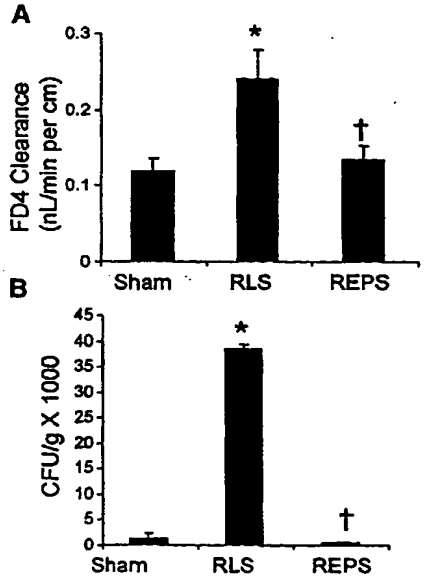

Fig. 1. Effect of resuscitation with Ringer lactate solution (RLS) or Ringer ethyl pyruvate solution (REPS) on ileal mucosal permeability (*A*) and bacterial translocation to mesenteric lymph nodes (MLN, *B*) assessed 4 h after the end of shock. Three groups of mice were studied. Sham mice (n = 7) were subjected to anesthesia and vascular cannulation but not to hemorrhagic shock. RLS (n = 12) were subjected to hemorrhagic shock for 2 h and resuscitated with RLS. REPS (n = 8) were subjected to hemorrhagic shock for 2 h and resuscitated with REPS. Results are means ± SE. *P < 0.05 vs. Sham group. †P < 0.05 vs. RLS group. FD-4, fluorescein isothiocyanate dextran with molecular mass of 4,000 Da; CFU, colony-forming units.

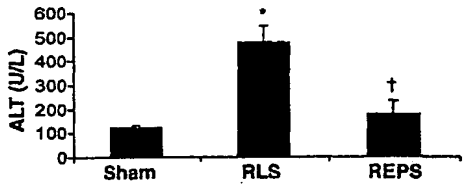

Fig. 2. Effect of resuscitation with RLS or REPS on circulating alanine aminotransferase (ALT) concentration assessed 4 h after the end of shock. Groups and symbols are the same as in Fig. 1.

Expression of IL-6 increased in the RLS group relative to the Sham group in samples of hepatic, ileal mucosal, and colonic mucosal tissue (Fig. 5, *A–C*, respectively). Substituting REPS for RLS as the resuscitation fluid decreased IL-6 expression in all three tissues.

Ileal mucosal iNOS expression was relatively unchanged after HS/R in both the REPS and RLS groups, whereas colonic mucosal iNOS expression actually tended to decrease in the RLS (but not the REPS) group relative to sham-hemorrhaged controls (data not shown). Ileal mucosal TNF expression increased in both the RLS and REPS groups, whereas expression of this cytokine was unchanged in samples of colonic mucosa (data not shown). Ileal and colonic mucosal COX-2 expression was lower after HS/R in the REPS group compared with the RLS group (data not shown).

Immunohistochemistry. HS/R was associated with an apparent increase in hepatic iNOS expression in the RLS group but not the REPS group (Fig. 6). These findings were consistent with the results obtained by semiquantitative RT-PCR.

DISCUSSION

In 1904, Holleman (14) reported that pyruvate and related α-keto acids with the general structure R—CO—COOH reduce H_2O_2 nonenzymatically in a reaction that yields carbon dioxide and water. In the case of pyruvic acid, this oxidative decarboxylation reaction can be written as follows: $CH_3COCOO^- + H_2O_2 \rightarrow CH_3COO^- + H_2O + CO_2$. Subsequent studies verified that this reaction is rapid and stoichiometric (5, 28). In addition to scavenging H_2O_2, pyruvate is also capable of scavenging OH · (11). In 1991, Salahudeen et al. (39) reported that intravenous infusion of sodium pyruvate protects rats from renal parenchymal injury induced by injecting H_2O_2 into the renal artery. After the publication of that paper, a number of other investigators reported that treatment with pyruvate could protect animals from the deleterious effects of a variety of conditions thought to be mediated by ROS, including myocardial I/R (4, 7, 8, 11, 35), intestinal I/R (6), and HS/R (30).

Despite these findings, pyruvate has not been developed as a therapeutic agent, probably because it is relatively unstable in solution (54) and is capable of forming potentially toxic byproducts (31). Ethyl pyruvate, a simple derivative of pyruvate, is more stable than the parent compound (Alfred Ajami, Xanthus, personal communication). Nevertheless, this compound has not been extensively evaluated as a therapeutic agent. One reason for the paucity of prior work with ethyl pyruvate may relate to its poor solubility (0.25% wt/vol in saline). Sims et al. (48), however, discovered that the use of a balanced, calcium-containing salt solution (analogous to RLS) markedly in-

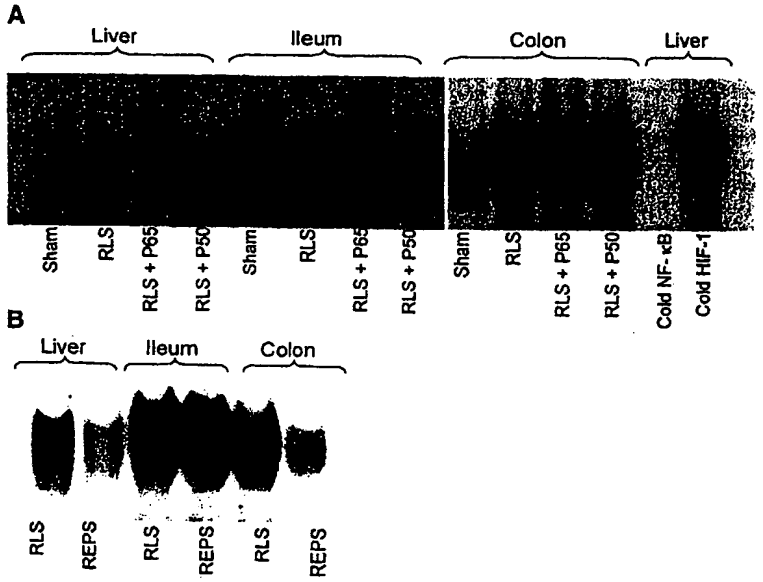

Fig. 3. Effects of HS/R on DNA binding of nuclear factor (NF)-κB in liver, ileal mucosa, and colonic mucosa. Electrophoretic mobility shift assay was performed using nuclear extracts prepared from tissues obtained 4 h after starting resuscitation or 4 h after the end of the sham procedure. *A*: comparison of samples obtained from animals in the Sham and RLS groups and depiction of the results of supershift assays using antibodies against the p65 and p50 subunits of NF-κB. Shown as well are the results of cold competition experiments using a 100-fold molar excess of either unlabeled (specific) NF-κB duplex oligonucleotide or unlabeled (nonspecific) hypoxia-inducible factor (HIF)-1 duplex oligonucleotide. *B*: comparison of samples obtained from animals in the RLS and REPS groups.

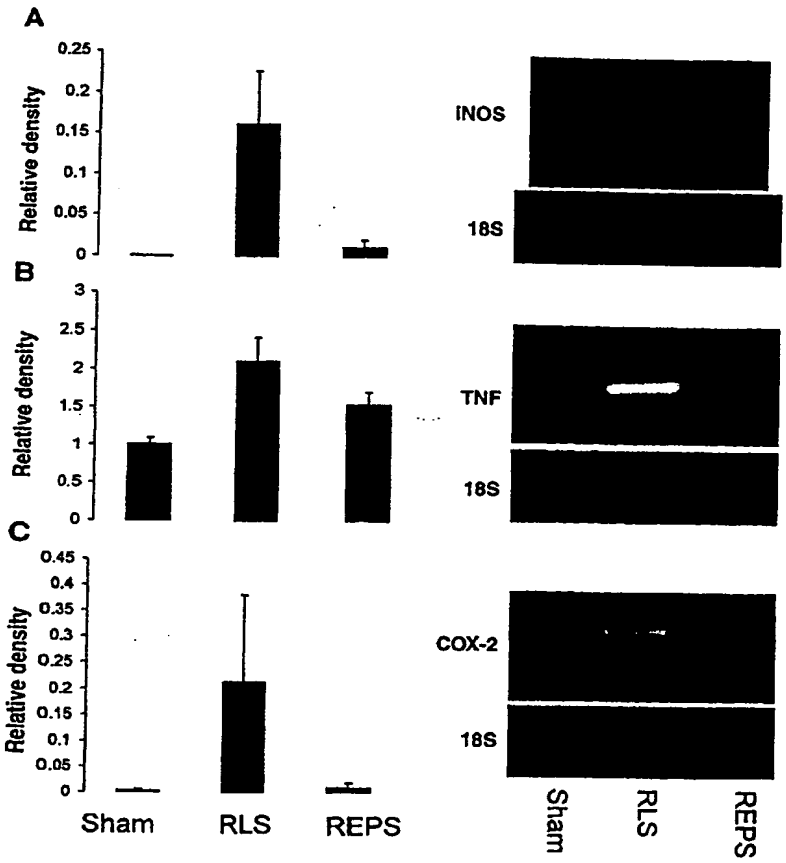

Fig. 4. Hepatic expression of inducible nitric oxide synthase (iNOS, *A*), tumor necrosis factor (TNF, *B*), and cyclooxygenase (COX)-2 (*C*) mRNA in mice subjected to hemorrhagic shock and resuscitation (HS/R) or the sham procedure. Results were obtained using semiquantitative RT-PCR as described in MATERIALS AND METHODS. Bands visualized after agarose gel electrophoresis of PCR reaction products were scanned using a NucleoVision imaging workstation and quantified using GelExpert release 3.5. Data in bar graphs are means ± SE (n = 3 or 4 per condition). Representative gels are depicted.

creases the solubility of ethyl pyruvate to 1.5% (wt/vol) or 130 mM. The basis for the increased solubility of ethyl pyruvate in a Ringer-type solution is thought to be stabilization of the enolate form of ethyl pyruvate by Ca^{2+} (48).

A formulation of ethyl pyruvate in a Ringer-type balanced salt solution, i.e., REPS, has been evaluated in two prior studies. In the first, Sims et al. (48) showed that pre- and posttreatment of rats subjected to mesenteric I/R largely prevented structural damage to the intestinal mucosal caused by this stress and also significantly ameliorated the development of gut mucosal hyperpermeability after reperfusion. Subsequently, in a study using a rat model of HS/R, Tawadrous et al. (51) showed that resuscitating rats with REPS instead of RLS prevented the development of mucosal hyperpermeability and ameliorated lipid peroxidation, a marker of ROS-mediated stress, in liver and gut.

In the present study, we have both confirmed and substantially extended the observations made in the prior study of REPS as a resuscitation fluid for HS/R. The differences between our laboratory's earlier study and the present one can be summarized as follows. First, we used a rat model of hemorrhagic shock in the study by Tawadrous et al. (51), whereas for the studies described herein we used a model of hemorrhagic shock in mice. Second, in the study by Tawadrous et al. (51), we showed that resuscitation with REPS prolongs survival during the first 4 h after resuscitation from shock, whereas we are now able to report that resuscitation with REPS instead of RLS improves permanent survival in a murine model of HS/R. Third, as in the previous study by Tawadrous et al. (51), we showed that resuscitation from hemorrhagic shock with REPS instead of RLS significantly ameliorated ileal mucosal hyperpermeability to FD-4. Hyperpermeability to hydrophilic macromolecules, however, is only one manifestation of gut barrier dysfunction. However, another important feature of gut barrier dysfunction associated with HS/R is increased bacterial translocation to MLN (2). It is noteworthy, therefore, that in the present study, we found that resuscitation with REPS instead of RLS abrogated bacterial translocation. This finding is consistent with data from previous studies (9, 10)

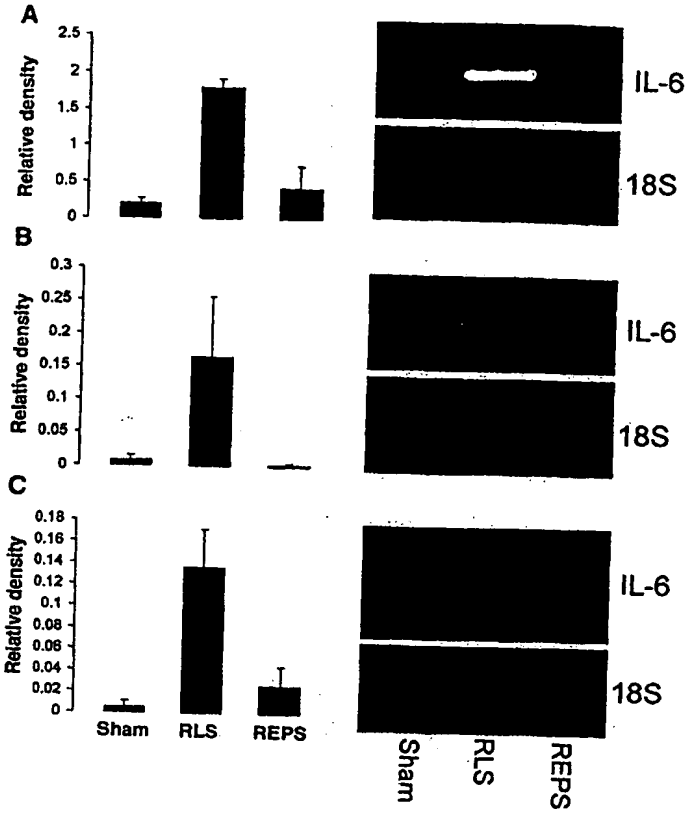

Fig. 5. Expression of interleukin (IL)-6 mRNA in samples of hepatic (*A*), ileal mucosal (*B*), and colonic mucosal (*C*) tissue from mice subjected to HS/R or the sham procedure. Results were obtained as described in Fig. 4. Data in bar graphs are means ± SE (n = 4 per condition). Representative gels are depicted.

showing that various ROS scavengers are capable of ameliorating bacterial translocation caused by hemorrhagic shock. Fourth, in the present study, we also showed that resuscitation with REPS ameliorated HS/R-induced hepatocellular damage. This observation is consistent with data obtained in two other recent studies, wherein treatment before resuscitation with the ROS scavengers tempol (32) and *N*-2-mercaptopropionyl glycine (17) ameliorated hepatocellular injury associated with HS/R in rodents. Fifth, in the present study, we carried out experiments to examine the effects of REPS on various aspects of the inflammatory response after HS/R.

Previous investigators have shown that HS/R is associated with activation of the transcription factor NF-κB in various organs and tissues, such as liver (1, 13), lung (13), pulmonary mononuclear cells (45), heart (27), and kidney (25). Our results, showing HS/R-induced activation of NF-κB in liver and colonic mucosa, are consistent with these observations. Interestingly, we found that NF-κB was constitutively activated in ileal mucosa, and HS/R caused little or no change in the degree of NF-κB DNA binding in this tissue. A high basal level of ileal mucosal NF-κB activation was previously described by Pritts et al. (36). To confirm the specificity of the EMSA for NF-κB, we carried out both cold competition and supershift assays. As expected, competition with an excess of unlabeled NF-κB probe eliminated binding by the ^{32}P-labeled NF-κB consensus duplex oligonucleotide, whereas addition of an excess quantity of an unlabeled irrelevant duplex oligonucleotide had no effect on binding by the hot NF-κB probe. This observation supports the specificity of the EMSA for NF-κB DNA binding. We observed clear evidence of a supershift when extracts were preincubated with a commercially available antibody against p65. This finding is in agreement with previously reported data reported by Pritts et al. (36) and suggests that the detected NF-κB dimers contained p65. However, we were unable to demonstrate clear evidence of a supershift when extracts were preincubated with an antibody against p50. Because this finding is inconsistent with reports from several other laboratories, we carried out similar studies using a different anti-p50 antibody from another supplier. Similar results, however, were obtained (data not shown). We are unable to determine whether this observation represents a problem with the antibodies we used or actual evidence

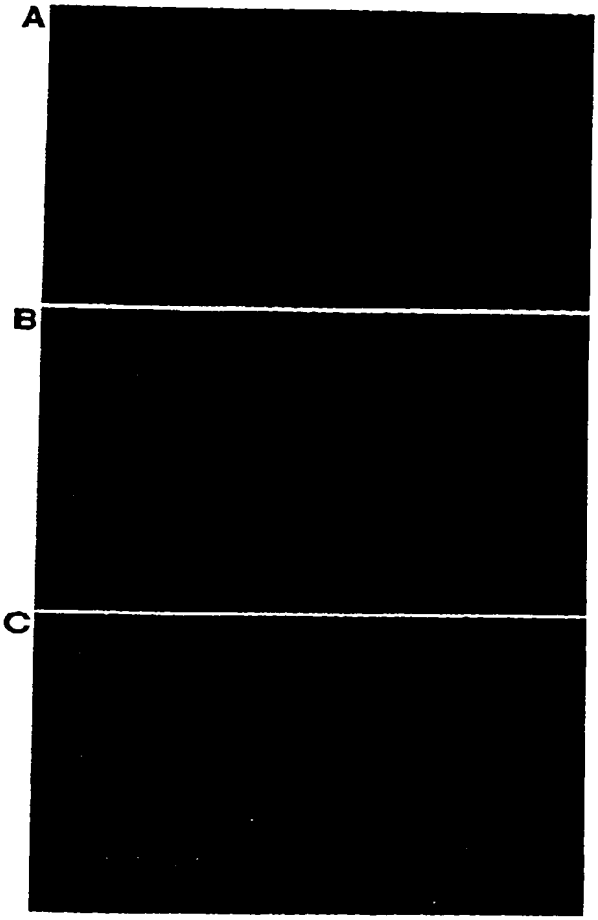

Fig. 6. Expression of iNOS in hepatic tissue samples from mice subjected to the sham procedure (*A*), HS/R with RLS (*B*), or HS/R with REPS (*C*). Green areas indicate immunoreactive iNOS. Blue areas have been stained with 4′,6-diamidino-2-phenylindole dihydrochloride, which binds to DNA and delineates nuclei. Original magnification was ×20 for all the images.

that we detected DNA binding by NF-κB-like constructs without a p50 subunit.

The transcriptionally active form of NF-κB is a homo- or heterodimer made up of various proteins belonging to the NF-κB family. These proteins include p50, p65 (RelA), c-Rel, p52, and RelB (3). In resting cells, however, these homo- or heterodimeric forms of NF-κB exist in the cytoplasm in an inactive form due to binding by a third inhibitory protein called IκB (3). Upon stimulation of the cell by a proinflammatory trigger, IκB is phosphorylated on two key serine residues, targeting IκB for ubiquination and subsequent proteosomal degradation. Phosphorylation of IκB is thought be mediated by various IκB kinases (IKKs) (18). Phosphorylation and degradation of IκB permit translocation of the transcriptionally active (dimeric) form of NF-κB into the nucleus and subsequent binding of the transcription factor to *cis*-acting elements in the promoter regions of various NF-κB-responsive genes.

Although the upstream events that lead to IKK activation are unclear (and probably differ depending on the inciting proinflammatory stimulus), it has been proposed that ROS are important in this process. Several lines of evidence support this view. First, numerous studies have shown that providing an exogenous source of ROS (e.g., by adding H_2O_2 to the medium for cultured cells) can trigger activation of NF-κB (23, 38, 40). Second, stimulating cells with various proinflammatory substances (e.g., TNF) leads to endogenous production of ROS (41). Third, various compounds with known antioxidant activity, such as *N*-acetylcysteine and pyrrolidine dithiocarbamate (PDTC), have been shown to block activation of NF-κB in cultured cells, not only by exogenous ROS but also by other proinflammatory stimuli (34, 41). Fourth, cytokine-stimulated activation of NF-κB tends to be exaggerated when cells are pretreated with an agent, such as buthionine sulfoximine, that depletes intracellular levels of glutathione, an important endogenous ROS scavenger (43). Finally, certain ROS scavengers, such as PDTC and dimethylthiourea, have been shown to block NF-κB activation in vivo as well (22, 49). Indeed, previous studies have shown that HS/R-induced NF-κB activation is downregulated by agents that either scavenge ROS or block their synthesis, including allopurinol (45–47) and IRFI-042 (a vitamin E analog) (1). Our results are consistent with these observations, since we found that resuscitation with a solution containing the ROS scavenger ethyl pyruvate (51, 53) downregulated NF-κB activation in liver and colonic mucosa in mice subjected to HS/R.

Previous studies (13, 46, 47, 50) have shown that HS/R leads to activation of a number of stress-related and proinflammatory genes. Although it is likely that more than one mechanism is responsible for activation of these genes after HS/R, one important factor appears to be increased formation of ROS and activation of various redox-sensitive signaling cascades. Treatment of experimental animals with ROS scavengers has been shown to inhibit HS/R-mediated upregulation of TNF (1, 17, 42, 50), IL-6 (50), and macrophage inflammatory protein-2 (47). In the present study, we confirmed and extended these findings by showing that treatment with REPS downregulated HS/R-induced expression of TNF, iNOS, COX-2, and IL-6 to varying degrees, depending on the tissue examined. It is noteworthy that resuscitation with REPS significantly inhibited HS/R-induced upregulation of IL-6 in ileal mucosa, since NF-κB activation in this tissue was largely unchanged by HS/R (irrespective of whether RLS or REPS was used for resuscitation). These data suggest that ethyl pyruvate is capable of modulating one or more signal transduction pathways not involving

NF-κB that are important in control of the inflammatory response to HS/R in at least some tissues.

Although Tawadrous et al. (51) and others (53) previously showed that ethyl pyruvate is capable of functioning as an ROS scavenger in vivo or in vitro, the possibility exists that this molecule might be beneficial in hemorrhagic shock for other reasons. For example, it is possible that when used as a component of resuscitation fluid for hemorrhagic shock, ethyl pyruvate functions as a metabolic substrate to decrease the cytosolic [NADH]/[NAD^+] ratio and maintain the cellular phosphorylation potential, [ATP]/[ADP][P_i]; this mechanism has been proposed to explain some of the beneficial effects of the parent compound pyruvate in a porcine model of hemorrhagic shock (30). However, it also possible that ethyl pyruvate functions in ways that are quite distinct from those of pyruvate. This notion is supported by studies showing that a related pyruvate ester, methyl pyruvate, stimulates insulin secretion by isolated pancreatic islets (29, 56), whereas pyruvate is not insulinogenic (44). To explain the differential effects of these two closely related compounds, it was speculated that the more lipophilic compound, methyl pyruvate, might penetrate the mitochondrial matrix better than pyruvate and thereby support supranormal rates of ATP production. However, recently reported data refute this hypothesis and suggest that pyruvate and methyl pyruvate have distinct biochemical effects in pancreatic β-cells that are unrelated to ATP biosynthesis (21). It is unknown at present whether ethyl pyruvate also has biochemical actions that are clearly distinct from those of pyruvate. However, in an earlier study (48), we observed that ethyl pyruvate provided better protection than pyruvate against gut mucosal damage caused by mesenteric I/R in rats.

In summary, we showed herein that HS/R in mice is associated with intestinal barrier dysfunction, hepatocellular injury, and increased expression of a number of stress-related and/or proinflammatory genes. These effects of HS/R were all attenuated, if not blocked completely, when the experimental animals were resuscitated with REPS, instead of a conventional crystalloid resuscitation fluid. These data support the view that REPS warrants further evaluation as a therapeutic agent for the prevention of organ injury and systemic inflammation after resuscitation from hemorrhagic shock.

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